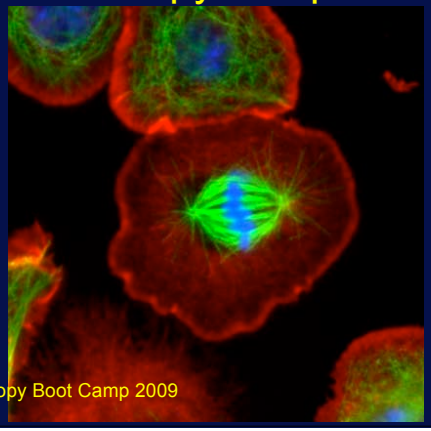
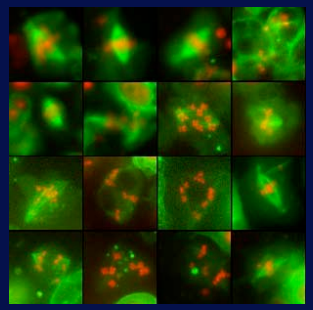
Fluorescence, Fluorescent microscopy and probes



Nico Stuurman
UCSF Microscopy Boot Camp 2009

Fluorescence in live cells

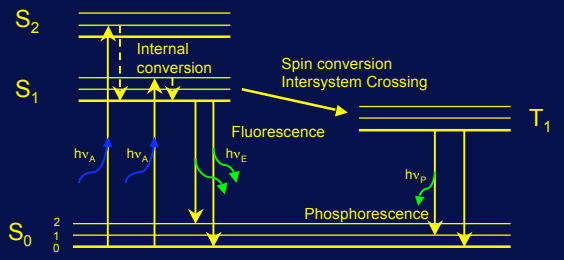


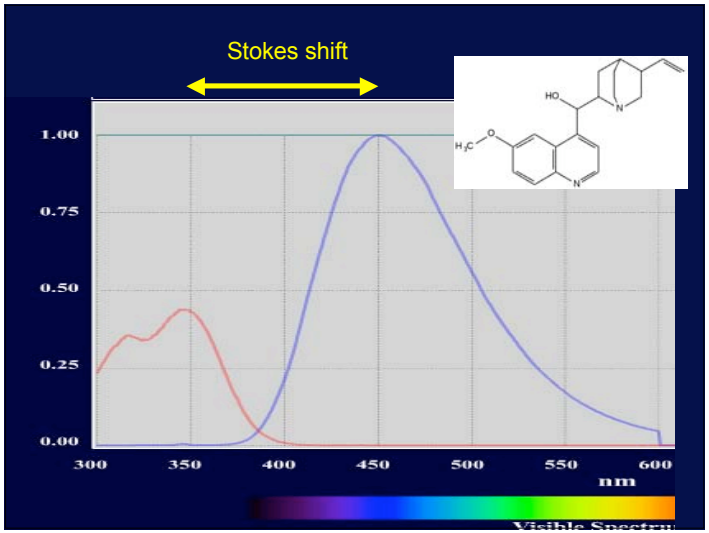
What is it?

Sir John Frederick William Herschel, 1854: Though perfectly transparent and colorless when held between the eye and the light, or a white object, it yet exhibits in certain aspects, and under certain incidences of the light, an extremely vivid and beautiful celestial blue colour, which, from the circumstances of its occurrence, would seem to originate in those strata which the light first penetrates the liquid…..

Fluorescence

Jablonski diagram



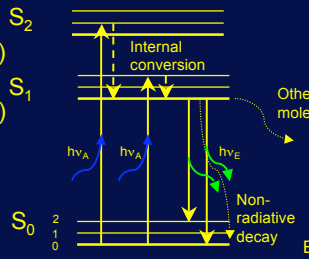


A matter of time

10^{-15}s (300nm)

10^{-12}s (300μm)

10^{-8}s (3m)

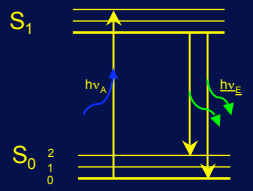


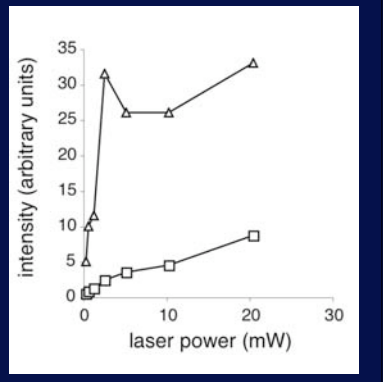
Absorption coefficient: frequency of absorption of photons

QE: ratio of absorbed and emitted photons

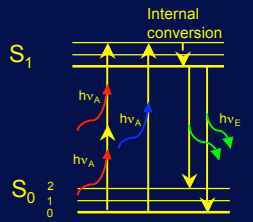
Brightness: determined by absorption coefficient and QE

Saturation



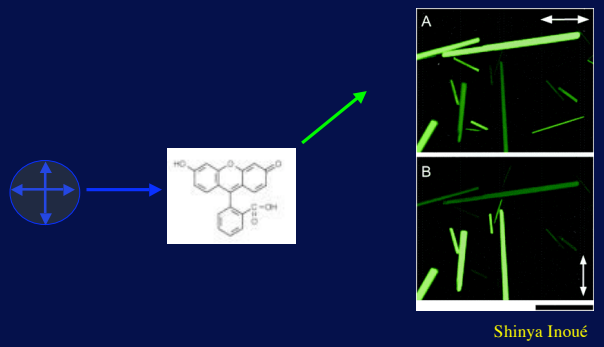


Multi-photon excitation



Brad Amos, MRC, Cambridge

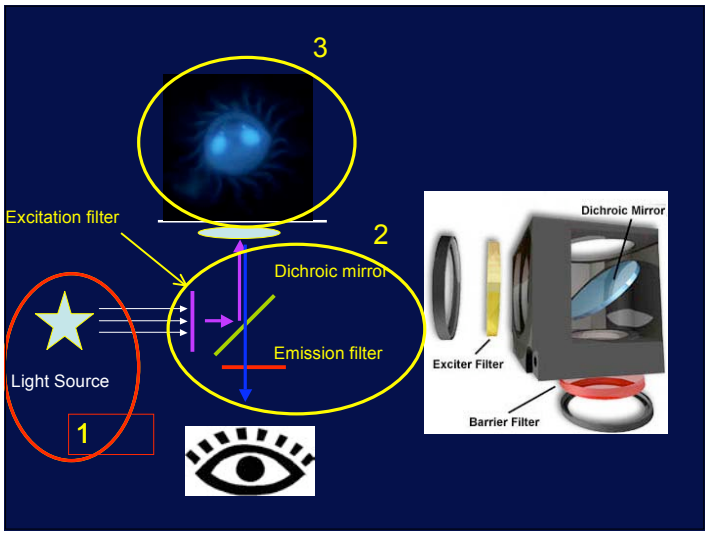
Polarization/Anisotropy



Shinya Inoué

Why use Fluorescence probes?

- Sensitivity
- Specificity
- Analysis of location and quantity of a single component in a complex mixture
- Detection of small quantities of fluorophores and fluorescent objects below the resolution limit
- Environmental sensitivity
- Does not rely on physical properties of the specimen for contrast generation

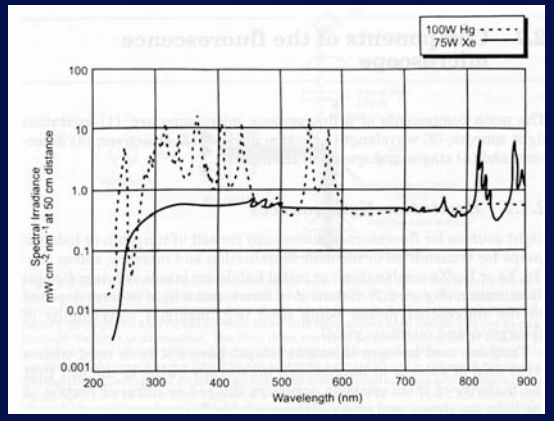


Commonly Used Light Sources for Fluorescence Microscopy

•Mercury arc lamp
•Xenon arc lamp
•Metal Halide doped Hg-Xe Arc

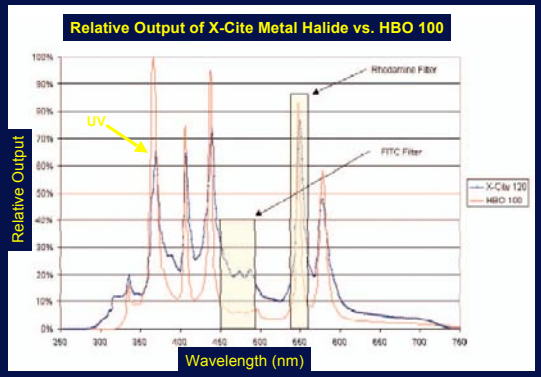
•LASERs
(most often used for confocal, TIRF)

Light Sources: Mercury & Xenon Arc Lamps

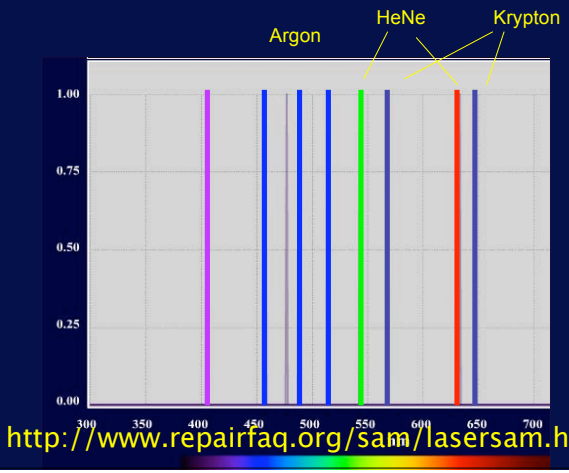


Metal Halide doped Hg-Xe Arc

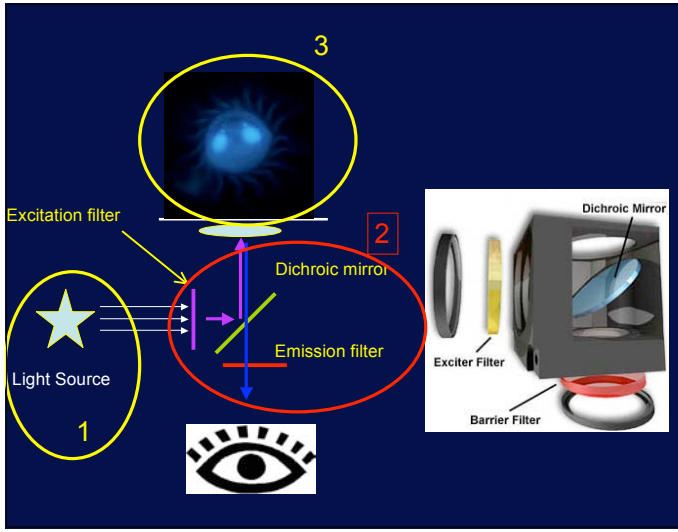
•Halogens decrease carbon deposits and slow deterioration of electrodes
•X-cite illuminator uses liquid light guide; minimal heat transfer



Laser lines



http://www.repairfaq.org/sam/lasersam.htm

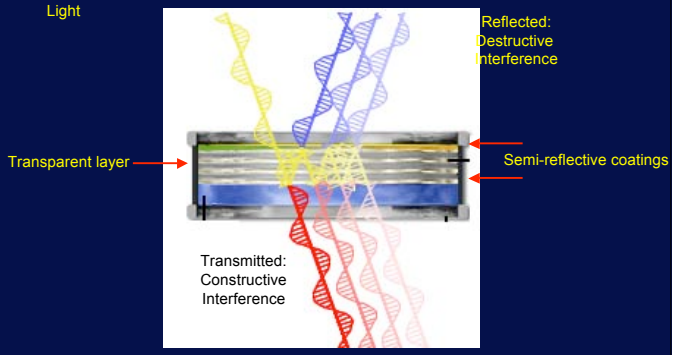


Filters

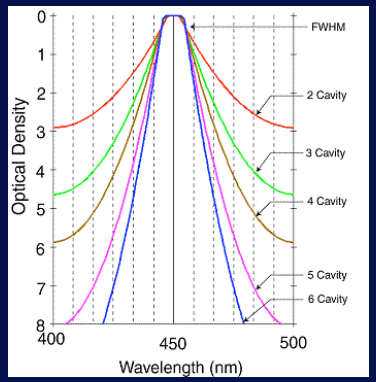
- Need to reject excitation light completely
- Need to be transparent for emitted light
- Need to match spectra of dyes
- Spectra of dyes:

www.zeiss.com/micro
probes.invitrogen.com/resources/spectraviewer/
www.mcb.arizona.edu/ipc/fret/

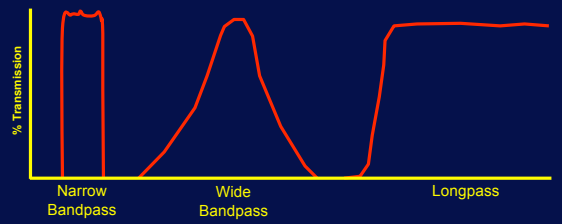
Interference Filter Design
(one cavity)



Interference Filter Design
(multiple cavities)



Filter Terminology



Center wavelength / Full bandwidth

525/50
525
525LP

Choose filters that maximize excitation and emission

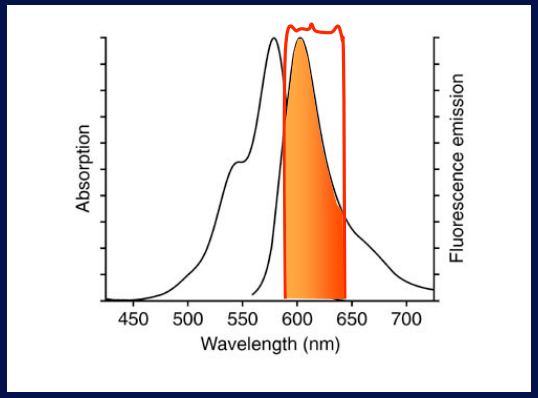


Image from www.probes.com

Choose filters that maximize excitation and emission

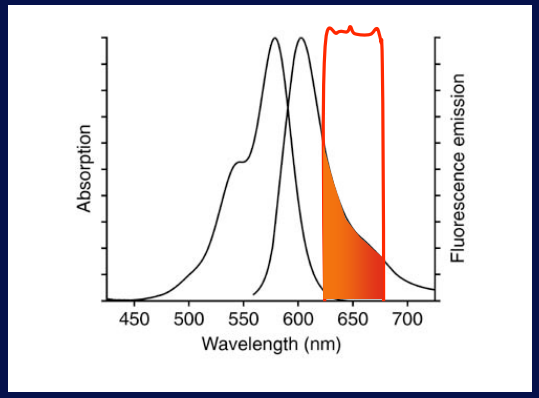
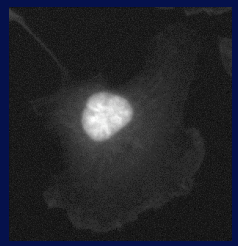
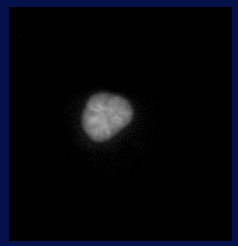


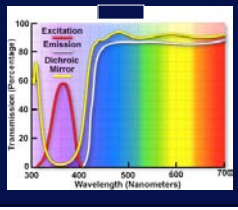
Image from www.probes.com

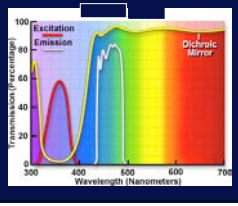
Choose filters that separate fluorophores



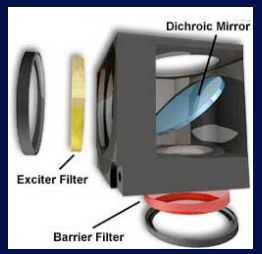


Two different UV filter sets

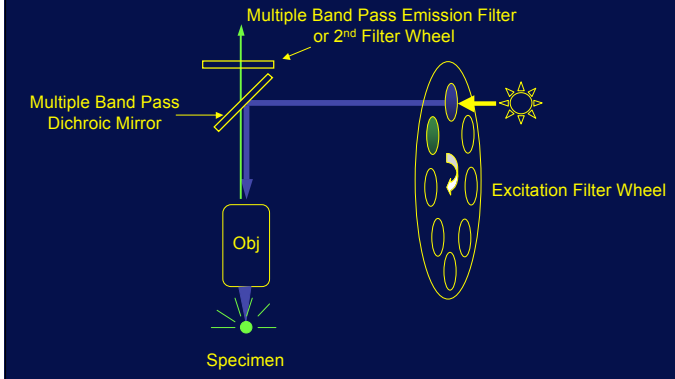




Filter cube (after Ploem)

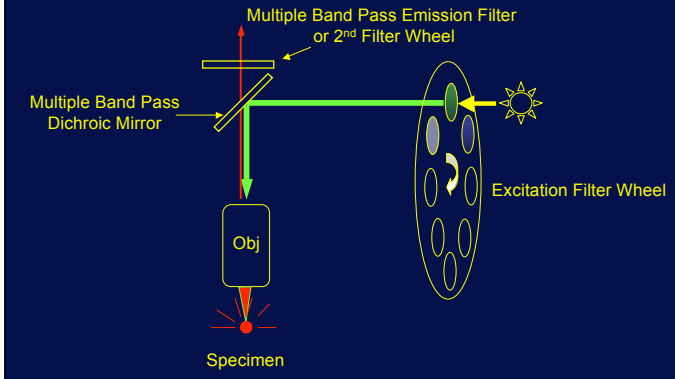


Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)



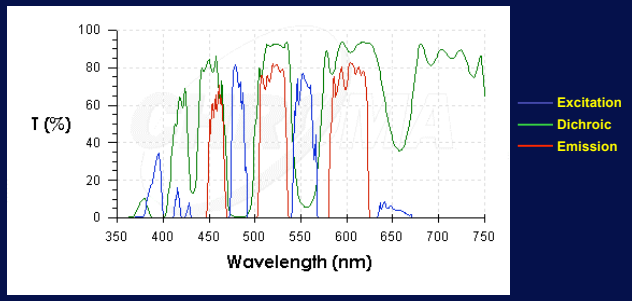
J.C. Waters

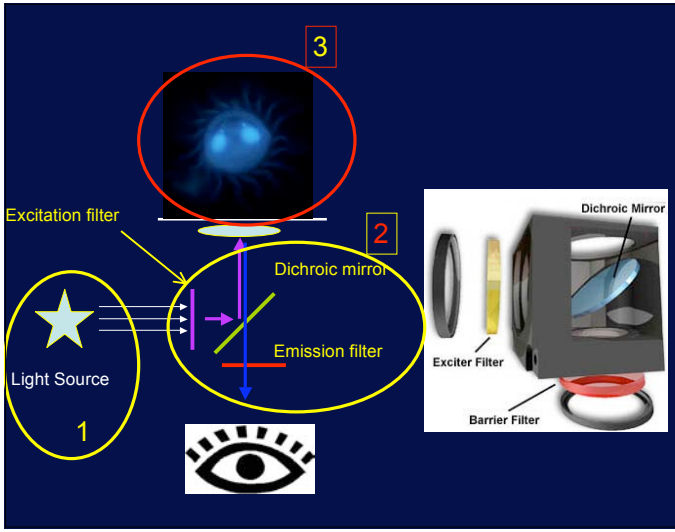
Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)



J.C. Waters

Multiple band pass filters

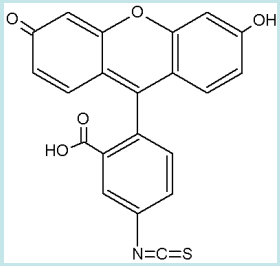




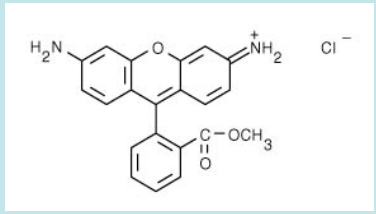
Types of fluorescent probes

- Immunofluorescence
- Fluorescent small molecules that bind specific cellular structures
  - –DNA intercalating dyes (DAPI)
- Fluorescently labeled small molecules that bind specific cellular structures
  - –Fluorescent phalloidin or taxol
- Fluorescently labeled proteins
- Fluorescent proteins (GFP)
- Genetically encoded tags binding fluorescent small molecules

The ‘classic’ dyes…

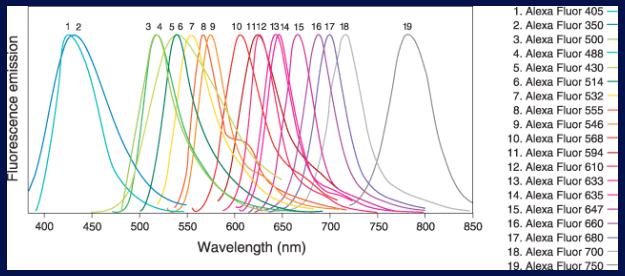


FITC



Rhodamine

The Alexa Series Emission Spectra



Molecular Probes (www.probes.com)

Conjugation of organic dyes

Chemistry/Method

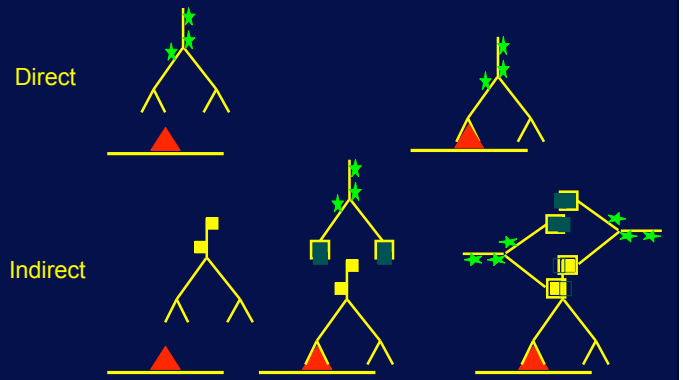
Amino groups (lysine): succinimidyl ester or isothocyanate

Example:
Dynein driven gliding of microtubules labelled with TMR on lysine residues.

Targets

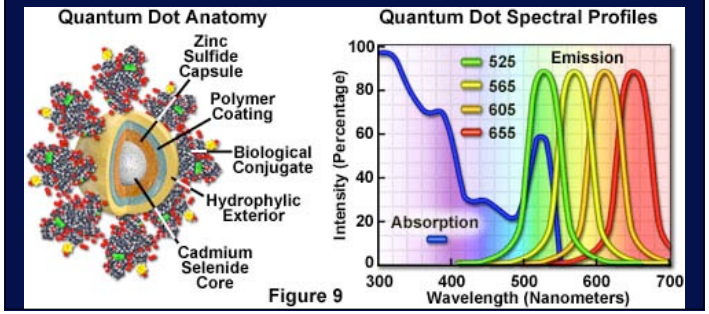
- Antibodies: direct/indirect labeling (Label density)
- Proteins: labeling site unspecific
- Small molecules, i.e. phalloidin, taxol

Immunofluorescence

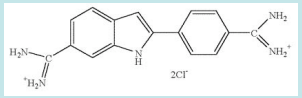


Quantum dots

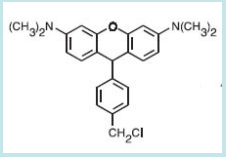
- nanometre-scale crystals composed of atoms of an inorganic semiconductor material



Small dyes targeting specific cellular targets

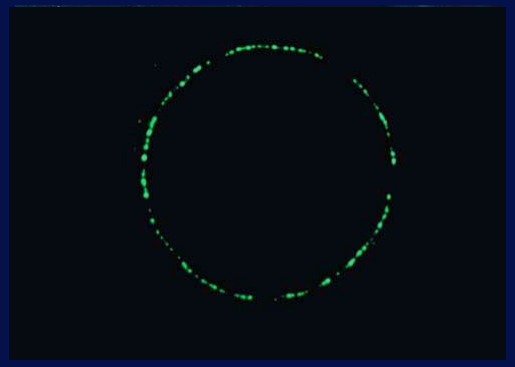


DAPI
Hoechst 33258
Hoechst 33342
~20 fold enhancement



Mitotracker
Oxidized in mitochondria in fluorescent compound

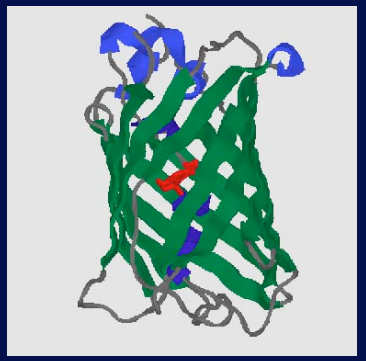
GFP-discovery

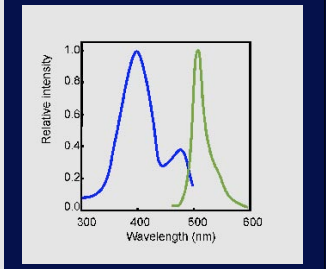


O. Shimomura
D.C. Prasher
M. Chalfie

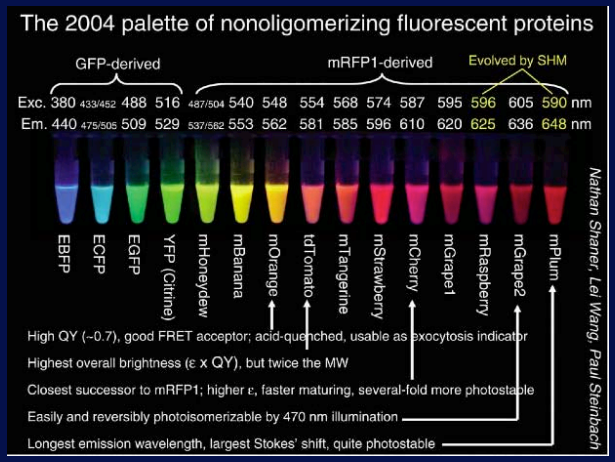
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

GFP structure





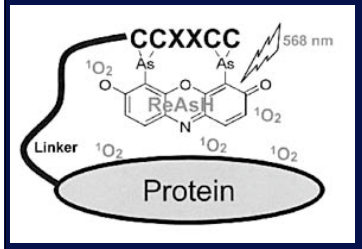
The 2004 palette of nonoligomerizing fluorescent proteins

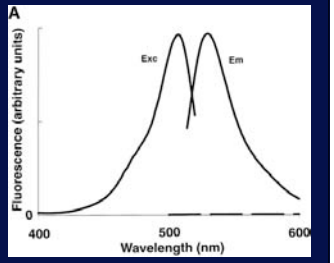


Roger Tsien

Other genetically encoded tags

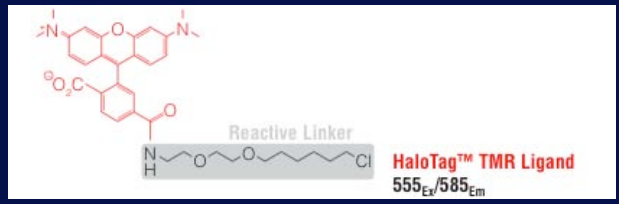
Biarsenical-tetracysteine labeling



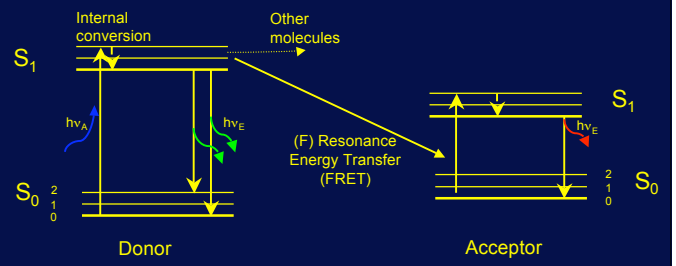


HaloTag

- Catalytically inactive mutant of a hydrolase that efficiently forms a covalent bond with HaloTag ligand (Promega)

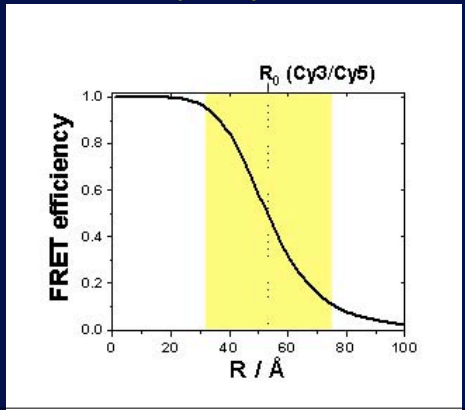


Quenching and FRET



Use FRET as molecular ruler

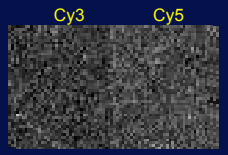
$E=R_0^6/(R_0^6 + R^6)$

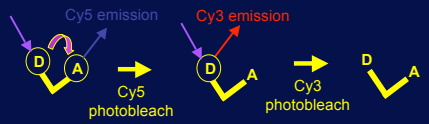


Evidence for single dye pair FRET

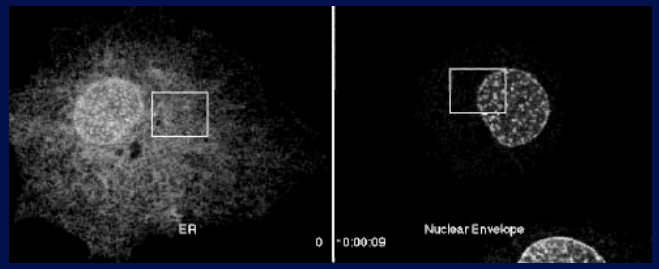
When Cy5 bleaches, Cy3 emission recovers

Kinesin showing ~100% FRET





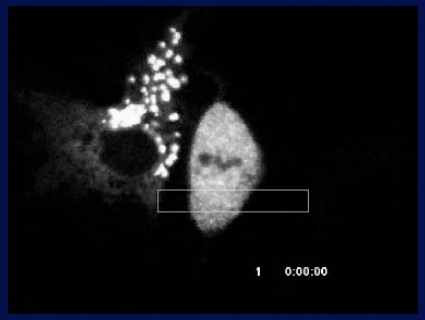
FRAP



Ellenberg et al., 1997 (Lippincott-Schwartz lab)

(Fluorescence Recovery After Photobleaching)

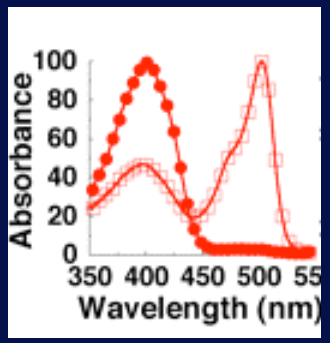
FLIP



Lippincott-Schwartz lab

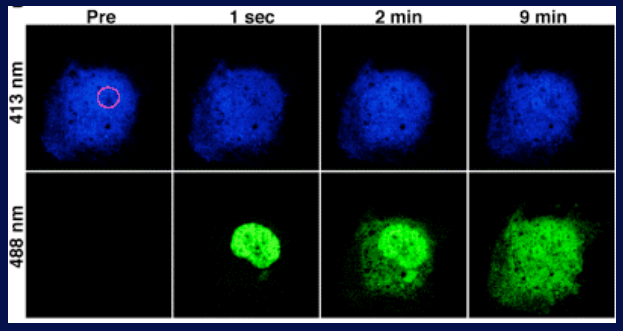
(Fluorescence Loss in Photobleaching)

Photo-activation (PA-GFP)

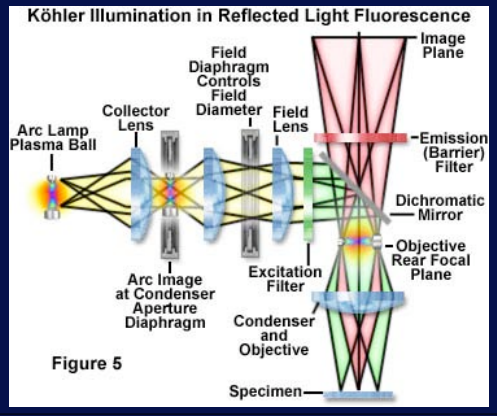


Excitation spectrum before (filled), and after (open) photoactivation

Photo-activation (PA-GFP)



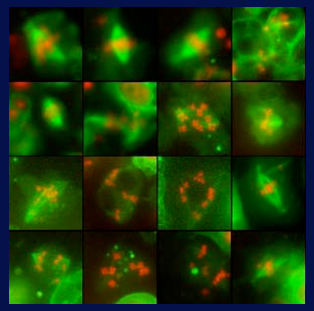
Koehler illumination



Lab samples:

- Drosophila S2 cells with various GFP-fusion proteins
- Stained tissue culture cells (multi-channel fluorescence)
- Fluorescent beads (visualize point spread function, registration shift)

THANKS!



Jennifer Waters
www.micro.magnet.fsu.edu
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